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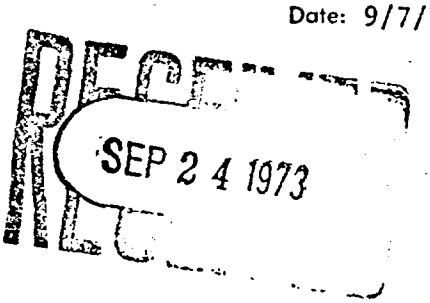
THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 9/7/73



1. Principal Investigator (give title and degrees):

Robert L. Volle, Ph.D., Professor
Edward G. Henderson, Ph.D., Associate Professor

2. Institution & address:

University of Connecticut
Health Center
Farmington, Connecticut 06032

3. Department(s) where research will be done or collaboration provided:

Pharmacology

4. Short title of study:

Molecular mechanisms underlying the development of tolerance to nicotine.

1003540364

5. Proposed starting date: January 1, 1974

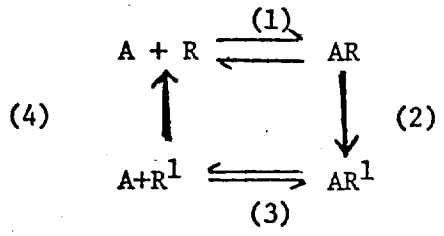
6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Our primary objective is to study the pharmacology of nicotine, lobeline and other nicotinic agents with the view of learning about central and peripheral toxicity to these drugs. Special emphasis will be placed on the study of the mechanism of tolerance to the nicotine drugs. It is well known that tolerance to nicotine develops when the compound is taken repeatedly. This is evident from the fact that confirmed tobacco smokers adapt to large amounts of the alkaloid while marked symptoms are exhibited by the tyro. A parallelism exists between this adaptive response to continued tobacco smoke and the adaptation or desensitization to nicotine which occurs at peripheral sites. A thorough examination of the process of desensitization at peripheral sites should be of great value in understanding central adaptive responses to nicotine. The proposed study will involve a further characterization of the mechanism of neuromuscular desensitization caused by lobeline and nicotine. The interaction of nicotinic agents with the neuromuscular junction and excitable tissues will be examined by radiochemical and electrophysiological techniques. It is expected that the proposed study will provide conclusive information as to the mechanism of lobeline's antagonistic actions toward nicotine and tobacco smoke and, in addition, will aid in understanding the underlying molecular mechanism involved in the desensitization process.

8. Brief statement of working hypothesis:

According to the cyclic hypothesis of the mechanism of receptor desensitization (Katz and Thesleff, 1957):



The interaction of an agonist molecule (A) with the end-plate receptor (R, reaction 1) leads to a depolarization. Desensitization results from the gradual transformation of the receptor into a non-reactive form R^1 (reaction 2) which reverts slowly to R after the withdrawal of the drug (reactions 3 and 4). Desensitization is generally associated with the repolarization of the end-plate membrane; but receptor desensitization with lobeline and nicotine can occur, under appropriate conditions, without causing a depolarization (Hancock and Henderson, 1972; Steinberg and Volle, 1972; Volle and Reynolds, 1973). In fact, it has been suggested that the use of depolarization alone as a measure of the drug effect is unsatisfactory (Rang, 1971). Earlier studies have demonstrated that the E_m of end-plates of depolarized skeletal muscles was not changed by addition of acetylcholine (ACh) but that the electrical conductance of the post-synaptic membrane was raised (Castillo and Katz, 1955) and the inward and outward movements of sodium and potassium were increased (Jenkinson and Nicholls, 1961). From this evidence it is clear that the interaction of nicotinic agents with the post-synaptic membrane caused an alteration of ionic exchange without depolarization. Whether or not the modification of a particular ionic exchange mechanism by the nicotinic agent is involved in the desensitization process will constitute the major emphasis of this project.

9. Details of experimental design and procedures (append extra pages as necessary)

It is well known that nicotine has a bi-phasic action on neuromuscular transmission (Thesleff, 1960). Whereas small doses of nicotine facilitate the transmission of impulses across the neuromuscular junction, a blockade of transmission occurs when larger doses of nicotine are applied. This inhibition of transmission is also bi-phasic: the initial block (phase I) is associated with depolarization and the late block (phase II) is associated with repolarization (Hancock and Henderson, 1972). Nicotine is also capable of being transported across the muscle cell membrane, altering calcium movement, and possibly exerting a direct effect on the muscle membrane (Weiss, 1966a, 1966b).

In order for neuromuscular transmission to occur, a depolarization or reduction of the resting potential of the motor nerve terminal must take place which results in the release of acetylcholine (ACh). ACh then diffuses across the synaptic gap and exerts its action (depolarization of the skeletal muscle fiber) at a specialized postsynaptic site (end-plate) on the skeletal muscle fiber membrane. When the depolarization of the end-plate attains critical values, the generation of muscle action potentials occurs. The conduction of action potentials by the muscle fiber membrane is regenerative in nature and, like other electrically excitable tissues, is associated with specific changes in sodium and potassium permeability. Whereas excitability of the muscle cell membrane is associated with very specific changes in sodium and potassium permeability, depolarization of the end-plate is generally considered to be accompanied by nonspecific ionic permeability changes.

Accordingly, nicotine could exert its effects on muscle by causing the release of [illegible] from a presynaptic storage site, by combining with specific receptors of the end-plate, by altering the permeability properties of the muscle membrane, by activating directly the contractile mechanism or by a combination of these mechanisms. There is very little known as to the sites where nicotine exerts its influence and what specific fundamental physiological processes are involved.

In contrast to nicotine, lobeline does not cause depolarization of the end-plate or the muscle fiber but it does cause an insurmountable blockade of the nicotine-induced depolarization (Steinberg and Volle, 1972; Hancock and Henderson, 1972; Volle and Reynolds, 1973). Nicotine reduces both quantal size and quantal content while lobeline reduces only quantal size, indicating that its effect is primarily post-synaptic.

Recent (unpublished) experiments in our laboratories indicate that lobeline (in the presence of d-tubocurarine) causes a frequency dependent depression of the twitch of directly stimulated frog sartorius muscle. Nicotine will also decrease the tension developed by directly stimulated muscle but the depression is transient and parallels the time course of the nicotine-induced depolarization-repolarization sequence. The nicotine induced twitch tension depression was blocked by d-tubocurarine while that caused by lobeline was not. This direct interaction of lobeline with the muscle cell membrane is suggestive of a local anesthetic type of interaction, since local anesthetics produce a similar response. Like lobeline, local anesthetics do not depolarize nerve or muscle fibers bathed in physiological solutions (Thesleff, 1956; Inoue and Frank, 1962) and block transmission at the myoneural junction (Thesleff, 1956; Castillo and Katz, 1957). The local anesthetic, procaine, has been demonstrated to interact with both pre- and postjunctional structures (Galindo, 1971), while only a postjunctional interaction of lobeline has been documented (Steinberg and Volle, 1972). In view of the similarities of the interactions of lobeline and local anesthetics with muscle cells and neuromuscular junctions, the fact that a prejunctional effect of lobeline has not been demonstrated is inconsistent. Therefore, it will be necessary to reexamine the effect of lobeline on neuromuscular transmission. The neuromuscular junction of both the frog sartorius and rat diaphragm will be impaled with microelectrodes and the parameters of transmitter release designated as quantal content (m) and quantal size (q) will be analyzed by measuring the variation of the amplitudes of end-plate potentials (EPP's) evoked by trains of stimulation of the sciatic nerve or phrenic nerve at rates of 5 to 20 impulses per second (Martin, 1966; Hubbard, et al., 1969). In the previous study of this type it was found that the decrease in EPP amplitude caused by lobeline (10^{-5}M to $2x10^{-5}$M) was due to an interference with the postjunctional action of the transmitter as evidenced by a decrease in quantal size (q) without significantly altering quantal content (m) (Steinberg and Volle, 1972). In the proposed study concentrations of lobeline ($\geq 5x10^{-5}$M) which produce a local anesthetic type block of muscle twitch will be employed. If the parallelism with local anesthetics persists, a decrease in both q and m would be expected.

In order to further evaluate the possibility of a local anesthetic type of interaction of lobeline with cell membranes, the effects of lobeline on the threshold for excitation of muscle cell membranes and membrane ionic conductance will be evaluated. It is characteristic of local anesthetic interaction with excitable tissues that the threshold for action potential generation is elevated in the presence of the local anesthetic. Therefore, the effects of lobeline on the directly stimulated muscle fiber will be evaluated and compared with typical local anesthetics. Methods to be employed will be similar to those previously described (Fatt and Katz, 1951; Castillo and Katz, 1955; Adrian, Constantin and Peachey, 1969). The end-plate and non-end-plate membrane will be impaled with two glass microelectrodes. One of the electrodes filled with 3M K^+-citrate will be used to pass an electrical current while the other will be used to record the resulting voltage displacement. Both the current applied and the voltage displacement will be monitored with a Tektronix 502A oscilloscope and recorded with a Lehigh Valley Electronix moving film camera. The current-voltage records obtained will be analyzed in the manner originally described by Hodgkin and Rushton (1946). The data obtained in this manner can be used to calculate the total membrane conductance (G_m). The G_m reflects the total ionic exchange under the specific conditions to be employed.

The depolarizing current necessary to cause a propagated action potential represents threshold for excitation. Current-voltage records and threshold stimulating currents will be measured before and after the application of lobeline, nicotine and local anesthetics. If lobeline modifies the measured parameters in the same way as typical local anesthetics, further evidence will be obtained to characterize the interaction of lobeline as local anesthetic-like.

An additional phase of this project will involve an examination of the mechanism responsible for the decreased rate of desensitization caused by depolarization of muscle cells (Magazanik and Vyskocil, 1970) and temperature reduction (Harris and Leach, 1968). It is suggested from these earlier studies that desensitization may involve the ionic channels of the membrane rather than or in addition to the receptors. Accordingly, a study will be made of the mechanism responsible for the decreased rate of desensitization to nicotine at low temperatures and in depolarized muscles. Both the reduced temperature and depolarization effects are suggestive of a Na^+-K^+-ATPase inhibition. Since cardiac glycosides also inhibit the Na^+-K^+-ATPase system, the effects of these agents on the nicotine-induced depolarization-repolarization sequence will be measured and compared with the effects of low temperature and K^+-induced depolarization. The ability of lobeline to produce desensitization at low temperatures and in depolarized muscles will also be examined by the aforementioned electrophysiological methods.

In frog sartorius muscle there are more than one end-plate or chemosensitive sites per muscle fiber. While in most mammalian skeletal muscle fibers, the end-plate region of the neuromuscular junction usually occupies only a small fraction of the entire membrane surface. However, when the nerve supply to the skeletal muscle is severed and allowed to degenerate, extrajunctional receptors to ACh develop along the entire membrane surface (Thesleff, 1960; Miledi, 1960; Henderson and Hancock, 1971). The denervated muscle, then provides us with a system of expanded chemosensitivity, i.e., a magnified end-plate, and provides a nearly ideal preparation for investigating the effects of nicotine and lobeline on the permeability of the end-plate and extrajunctional receptors to various inorganic ions.

Therefore, the normal frog sartorius and the denervated rat diaphragm muscle will be used to study the effects of nicotine and lobeline on end-plate phenomena. It has been demonstrated that it is possible to measure a significant increase in the rate of potassium (^{42}K) efflux induced by the application of nicotine to a normal frog sartorius muscle and that denervation does not intensify the response (Henderson and Hancock, 1971; Hancock and Henderson, 1972). In contrast, only the denervated mammalian muscle responds to nicotine with an increase in ^{42}K-efflux (Henderson and Hancock, 1971). The increased rate of ^{42}K-efflux in both preparations follows the same time course as the depolarization-repolarization sequence. Therefore, in normal physiological saline solutions it is not possible to dissociate the E_m change from the ionic flux changes.

Recently, it has been demonstrated that nicotine can produce receptor desensitization without depolarization by first applying the drug in either a sodium free medium or in a solution with elevated K^+ (Volle and Reynolds, 1973). The subsequent application of an equivalent or higher dose of nicotine in a physiological solution does not produce a depolarization equivalent to that which would be expected under normal conditions. It should be possible with this technique to measure the alterations of ionic exchange caused by nicotinic agents in the absence of depolarization.

A preliminary experiment demonstrating the effects of nicotine on the rate constant of ^{42}K-efflux from frog sartorius muscle is shown in fig. 1. Two muscles from a frog

were incubated in radioactive (^{42}K) Ringer's solution overnight and then the washout or efflux of the radioactive tracer was followed in isotope-free, sodium-free (Tris-substituted for NaCl) by methods previously described (Sjodin and Henderson, 1964; Henderson and Volle, 1972). At the time indicated by the first arrow nicotine (0.05 mM) was added to the solution bathing one of the muscles (•) while carbachol (0.1 mM) was added to the solution bathing the other muscle. Both nicotinic agents caused a significant increase of the rate constant for ^{42}K-efflux. At the time indicated by the second arrow the drugs were removed and the rate of efflux returned to control levels. The third and fourth arrows indicate the second addition of equivalent concentrations of the drugs. These experiments indicate that the stimulation of ^{42}K-efflux by nicotinic agents can occur in the absence of depolarization and that this stimulation of K-exchange was not desensitized since the degree of stimulation produced by the second application of the drugs was equivalent to the first.

These experiments yield only preliminary findings and many more with varying doses of nicotinic agents and varying exposure times will be needed in order to evaluate the interaction of nicotine with the end-plate potassium ionophore. Similar experiments will also be undertaken with strips of denervated rat diaphragm muscle in order to compare the responses of the two species. Additional experiments employing agents which specifically block K^+-exchange across the muscle cell membrane (e.g., 9-aminoacridine and Ba^{++}, Henderson and Volle, 1972) will be undertaken in order to determine whether or not the end-plate K^+ ionophore is the same as that of the surface membrane. Muscles will be bathed in a sodium-free solution containing one of the blocking agents and then the nicotinic agent will be added. If the stimulation of ^{42}K-efflux caused by nicotine is blocked by either 9-aminoacridine or Ba^{++}, it would be indicated that the end-plate K^+ channels are identical to those of the surface membrane.

Muscle cells depolarized by the elevation of extracellular K^+ concentration do not depolarize further after the addition of ACh but the end-plates respond to ACh with a change in electrical conductance and an increase in Na^+ and K^+ exchange (Castillo and Katz, 1955; Jenkinson and Nicholls, 1961). In addition, tetrodotoxin (TTX) and saxitoxin (STX) block conducted action potentials of nerve and muscle by specifically reducing the inward sodium current (Narahashi, Moore and Scott, 1964) while they do not prevent nicotinic agents from depolarizaing the end-plate membranes (Elmqvist and Feldman, 1965; Kao and Nishiyama, 1965). Like the examination of K^+-exchange, ^{22}Na-exchange will also be measured in this study under the aforementioned conditions. These experiments will allow us to determine whether or not the Na^+-ionophore of the end-plate membrane is the same as that of the surface membrane and to determine the contribution of sodium to the nicotine-induced desensitization phenomenon. The specific method of measuring ^{22}Na-exchange will be with procedures previously described (Mullins and Frumento, 1963; Sjodin and Beauge, 1968; Henderson and Volle, 1972).

These radiochemical techniques will also be employed to further evaluate the possible local anesthetic response to lobeline, since it has recently been demonstrated that local anesthetics block ^{42}K-exchange in resting muscle cells (Henderson and Volle, 1973). The local anesthetics butacaine and procaine were shown to block ^{42}K-exchange without depolarization in Cl^--containing media. In Cl^--free media the blockade of ^{42}K-exchange by local anesthetics was identical but the local anesthetics produced a significant depolarization. Depolarization is an expected consequence of decreased K^+-exchange and readily demonstrated when Cl^- conductance is diminished. Previous studies with lobeline involved measurements of E_m in Cl^--containing media (Hancock and Henderson, 1972; Volle and Reynolds, 1973). In these experiments no depolarization was observed with lobeline concentrations up to $5x10^{-5}M$. More recent preliminary experiments indicate that lobeline does cause a depolarization in Cl^--free media. By analogy, it would be predicted that K^+-exchange was depressed. The

ssibility of a lobeline-induced depression of K^+-exchange will be examined both by electrophysiological and radiochemical techniques and compared with the local anesthetic induced blockade of K^+-exchange.

In summary, this project will allow us to further evaluate the molecular mechanisms underlying the development of tolerance to nicotine and the antagonism between lobeline and nicotine. In addition, it is expected that the proposed study will allow us to determine whether a modification of the nicotinic receptor is involved in the desensitization process or an alteration of a particular ionophore. These studies of the interaction of nicotine and lobeline at peripheral sites will be of great value in understanding central adaptive responses to nicotine.

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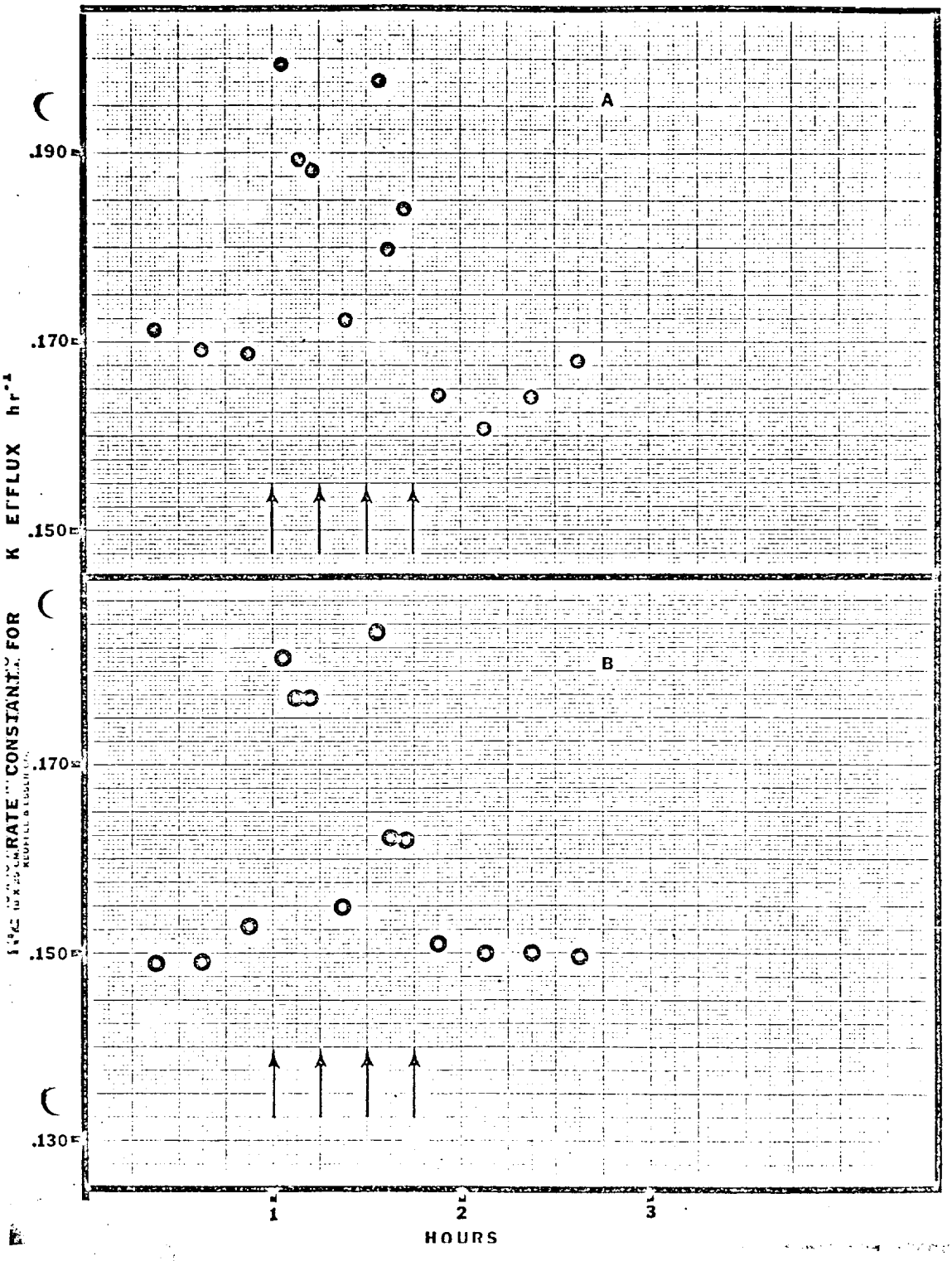
Fig. 1



10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The complete facilities of the Schools of Medicine and Dental Medicine will be available to the applicants. These include, The Health Center Library with a complete service for health related literature and a vivarium for quartering the animal species to be used in this study. The applicants have at their disposal laboratory and office space containing equipment for radioactivity and ion analysis and equipment for electrophysiological recording.

Major items of equipment available to the applicants include: a dual channel Auto-gamma spectrometer, three channel liquid scintillation spectrometer, atomic absorption spectrophotometer, flame photometric instrumentation, freezing point depression osmometer, desk top computer, dual channel oscilloscopes, square pulse generators, moving film recording camera, microelectrode puller and various high input impedance, capacity neutralization amplifiers. In addition, the facilities of the pharmacology department will also be available to the applicant. These include: a complete dark room, cold room, warm room and dishwasher-sterilization room.

11. Additional facilities required:
None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
R. L. Volle, Ph.D.	25	-
E. G. Henderson, Ph.D.	50	-
Technical		
Research Assistant III (To be appointed) Fringe Benefits (29%) ($2,755.)	100	12,255.
Sub-Total for A		12,255.
B. Consumable supplies (by major categories)		
Radioactive isotopes		3,000.
Animals		1,000.
Chemicals, glassware, etc.		800.
Electrophysiological and photographic supplies		500.
Sub-Total for B		5,300.
C. Other expenses (itemize)		
Principal investigator's travel for the presentation of findings at scientific meetings (Pharmacol. Soc. and FASEB)		400.
Maintenance of equipment		800.
Page charges and reprints of publications		400.
Sub-Total for C		1,600
Running Total of A + B + C		19,155.
D. Permanent equipment (itemize)		
None		
Sub-Total for D		-
E. Indirect costs (15% of A+B+C)		2,873.
Total request		22,028.

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	13,235.	5,000.	1,600.	-	2,975.	22,810.
Year 3	14,294	5,000.	1,600.	-	3,134.	24,028.

10035403Z4

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies of Sympathetic Ganglia Following Conditioning (Volle)	PHS Grant 5R01-NS07540-07	21,806.	9/1/73 - 8/31/74
Nicotinic Receptors in Muscle Membrane: A Study of Receptor Desensitization (Volle)	AMA-ERF (Terminal Year)	15,792.	8/1/73 - 7/31/74
The Pharmacology of Glycerol-treated Striated Muscle (Henderson)	University of Connecticut Research Foundation	2,900.	5/15/73 - 5/15/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Pharmacological Modification of Ionic Exchange (Henderson)	AMHL 17386-01	29,515.	1/1/74 - 12/31/74

it is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Robert L. Volle

Signature Robert L. Volle Date 9/7/73

Telephone 203 (Area Code) 674-2120 (Number) - (Extension)

Checks payable to

James C. Leming, Assistant Vice-President For Financial Affairs

Mailing address for checks

University of Connecticut Health Center

Farmington, Connecticut 06032

Responsible officer of institution

Typed Name Glenn W. Ferguson, President or Edward V. Gant, Provost

Title President or Provost

Signature Edward V. Gant Date 9/18/73

Telephone 203 (Area Code) 486-2338 or 203 (Area Code) 486-2418 (Number) (Extension)

10035403 25